

In the Specification

Kindly replace paragraph [0001] through [0026] with the following:

Related Application

This is a §371 of International Application No. PCT/FR2004/002673, with an international filing date of October 19, 2004 (WO 2005/040385, published May 6, 2005), which is based on French Patent Application No. 03/12250, filed October 20, 2003.

Technical Field

~~The invention~~This disclosure relates to ~~the area of~~ biology and, more particularly, to the preparation of double-stranded oligonucleotides ~~intended to be used~~for use in an RNA interference process (RNAi) ~~in order to induce the degradation of a target RNA.~~

Background

Interference RNA, also designated “RNAi”, or even co-suppression, has been detected in plants, where it was observed that the introduction of a long, double-stranded RNA corresponding to a gene induces ~~the specific~~ and efficacious repression of the expression of the targeted gene. The mechanism of this interference comprises ~~the degradation of the double-stranded RNA into short duplexes of oligonucleotides of approximately 20 to 22 nucleotides called siRNA’s.~~

Interference RNA has now been applied to mammals for specifically inhibiting ~~the~~ expression of genes for applications in functional genetics. In fact, siRNA’s permit ~~the identification of the function of genes detected by the sequencing of the human genome either in cellular culture models or in animal models and, in particular, in the mouse.~~ Interference RNA is also useful in the therapeutic area for the treatment or prevention of cancers, infectious diseases and, more generally, of diseases involving a heterologous or homologous mutated gene (S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschli (2001a), “Duplexes of 21-Nucleotide RNA’s Mediate

RNA Interference in Cultured Mammalian Cells”, Nature 411, 494-498; S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel and T. Tuschli (2001b), “Functional Anatomy of siRNA’s for Mediating Efficient RNAi in Drosophila melanogaster Embryo Lysate”, Embo J. 20 6877-6888).

siRNA’s are short sequences of double-stranded RNA that can be introduced into cells in the form of synthetic oligonucleotides or in the form of vectors permitting ~~the~~ expression of these siRNA’s. In this latter approach, siRNA is synthesized in the form of a precursor that is a shRNA (short hairpin RNAi), formed by a “rod loop” in which the rod represents the siRNA and the loop any sequence that will be degraded by the cellular RNAase’s, which will give ~~birth~~rise to the mature siRNA.

~~The implementation~~Implementation of siRNA expression vectors has numerous advantages, in particular, for applications of functional genetics. It allows double-stranded RNA to be expressed in a stable manner in cells and therefore to more readily inhibit ~~the~~ expression of proteins with a long half-life. In fact, synthetic siRNA’s have a half-life of 3 days in mammalian cells. Furthermore, siRNA’s are diluted in the course of cellular divisions.

It also permits long-term effects to be analyzed. On the other hand, it requires ~~the~~ establishment of lines expressing the construction in a stable manner, which presents several disadvantages. In particular, it is necessary to compare stable lines with each other, which is generally difficult to interpret because the cellular lines drift. On the other hand, it is impossible to study the proteins indispensable for the cell since their inhibition will block ~~the~~ proliferation of the cells and will thus prevent ~~the establishing~~establishment of the stable line. It is therefore indispensable to be able to induce the activity of siRNA. The term “induce the activity of siRNA” denotes being able to block and unblock its activity substantially at will. The term “activity of siRNA” denotes its capacity to recognize and induce ~~the~~ degradation of its target RNA.

The prior art describes a vector that permits the stable and inducible expression of siRNA. ~~This~~That system is based on ~~the~~ inhibition of the transcription of the precursor of siRNA. In fact, the vector, derived from the pSUPER vector (T. R. Brummelkamp et al, [www.sciencexpress.org/March 21, 2002/page 1/10. 1126/science.1068999](http://www.sciencexpress.org/March21,2002/page1/10.1126/science.1068999)), contains a known sequence, “the tetracycline operator” (“TET operator”), positioned between the promoter directing ~~the~~ transcription of the siRNA and the sequence coding ~~this~~that siRNA. In the presence of the protein “TET repressor,” the latter fixes itself on the “TET operator” and thus blocks ~~the~~ transcription of the siRNA. In the presence of doxycycline, the latter inhibits ~~the~~ fixation of the “TET repressor” on the “TET operator” and thus permits ~~the~~ transcription of the siRNA. This expression system engenders a relatively elevated background [~~background noise?~~]amount of activity/noise.

Moreover, the inducible systems based on the promoters functions very poorly in viral vectors because the viral promoters take precedence over the inducible promoters and the background [~~b. noise?~~]activity/noise is very elevated.

Summary

This invention relates to a method for inducing activity of an RNAi in cells including introducing TAT protein and a nucleic acid coding for sense and antisense sequences of an RNAi of interest into eukaryote cells, which sense and antisense sequences are separated by a nucleotide separating sequence including at least SEQ ID No. 1, coding for at least one nucleotide sequence contained in a TAR sequence for a time sufficient to be recognized by the TAT protein under conditions in which the nucleic acid is transcribed in RNA so that the transcribed separating sequence and the TAT protein form a complex inhibiting the activity of the RNAi of interest, and withdrawing the TAT protein to induce activity of the RNA.

This invention also relates to a cell or a line of cells transfected by the nucleic acid.

This invention further relates to a pharmaceutical composition including at least one nucleic acid and, optionally, a compatible excipient.

This invention still further relates to a pharmaceutical composition including a cell or line of cells and, optionally, a compatible excipient.

Brief Description of the Drawings

Fig. 1 is a Western blot that used a total extract with an antibody directed against the GFP or cellular tubulin to evaluate the quantity of proteins.

Detailed Description

~~The invention intends specifically to remedy~~method described herein remedies these disadvantages by ~~offering~~providing a system that permits the activity of a siRNA to be induced substantially at will. This goal is achieved by ~~the present invention by the use of the~~a protein couple TAT-sequence RNA TAR ~~in order~~ to block the activity of a siRNA in mammalian cells.

The principle ~~of the invention~~ is based on the interaction of TAT/TAR. In fact, the siRNA ~~must be~~is recognized in the cell by a protein complex called “RISC complex” (RNAi-Induced Silencing Complex) that causes it to undergo a maturation stage and ~~will use~~s it as a guide ~~in order~~ to recognize and degrade the target mRNA. The TAT protein can be used to block ~~this~~the latter stage.

The TAT protein of VIH plays a crucial part in viral replication. In fact, TAT regulates, as a transcription factor, ~~the~~ replication speed of the virus. TAT is a secreted protein capable of diffusing into non-infected cells and ~~of~~ preparing an environment propitious for viral infection. The TAT protein exists in two forms (71 and 101 amino acids) coded by two exons separated by the *env* gene. TAT exercises its function as activator of the promoter of VIH by binding to the RNA TAR sequence present at 5' of all the RNA's of the VIH.

~~The linking of~~ Linking TAT and of its transcriptional cellular co-activators to RNA TAR will stimulates ~~the~~ elongation of the transcription by augmenting the processivity of RNA polymerase II.

The presence of double-stranded RNA's in a cell represents a signal: It involves an RNaseIII called Dicer. This ribonuclease cleaves the long, double-stranded RNA's into small fragments of double-stranded RNA, the siRNA's, with a precise size and structure, in general 21 to 23 nucleotides. The duplex siRNA's then become associated with proteins ~~in order~~ to form a complex called RISC (for "*RNAi-Induced Silencing Complex*"). RISC is therefore a ribonucleoprotein complex containing a molecule of siRNA associated with proteins belonging to the Argonaut family. RISC guides the small siRNA's by virtue of the 5' phosphate end of a simple-stranded siRNA for the specific recognition of the target messenger RNA and catalyses its cleavage; ~~this~~. This reaction takes place in the cytoplasm. This terminates in the inhibition of the translation and thus in the inhibition of the expression of the target gene.

~~According to the invention the~~ The loop of the precursor of the siRNA (shRNA)[[,]] normally constituted by any sequence, is replaced by a nucleotide sequence coding for at least one nucleotide sequence contained in the TAR sequence, minimal and sufficient for being recognized by the TAT protein.

This sequence ~~referenced in the sequence list submitted in the annex under the number~~ is SEQ ID No. 1 and is composed of 11 nucleotides and has the following DNA banding pattern-~~concatenation~~: ATCTGAGCTCT (or AUCUGAGCUCU in its RNA form).

It is also possible to use, ~~in accordance with the invention,~~ a sequence coding for the entire wild or mutated TAR sequence or any fragment of it containing this minimal, non-mutated nucleotide sequence described above. This sequence (DNA or RNA) is named elsewhere in the separate

sequence text. The separate sequence ~~used in accordance with the invention~~ can be natural or synthetic.

Thus, in the presence of the TAT protein, the latter interacts with the TAR loop on the shRNA, and the interaction of the siRNA protein maturation complex (RISC complex) cannot be realized and the siRNA does not undergo the maturation stages ~~in~~-induced by this complex and, therefore, remains inactive, that is to say, in the shRNA form.

In the absence of the TAT protein, the TAR loop of shRNA is degraded and the resulting siRNA is assessable to the RISC maturation complex and the degradation process of the target RNA by the RNAi can thus be carried out.

Therefore, the ~~invention has as subject matter~~ a method for inducing the activity of an RNAi in cells includes which:

- The TAT protein and a nucleic acid coding for the sense and antisense sequences of an RNAi of interest are introduced into eukaryote cells, which sense and antisense sequences are separated by a nucleotide sequence comprising at least ~~the sequence referenced under number SEQ I No. 1 in the sequence list supplied as an annex~~, coding for at least one nucleotide sequence contained in the TAR sequence, minimal and sufficient for being recognized by the TAT protein under conditions in which the nucleic acid is transcribed in RNA, which transcribed separating sequence and the TAT protein form a complex inhibiting the activity of the RNAi of interest;
- The activity of the RNAi is induced by withdrawing the TAT protein.

The separating sequence can ~~be constituted by~~comprise any nucleotide sequence provided that it comprises at least ~~the SEQ ID No. 1 sequence~~. The separating sequence is advantageously

constituted by ~~of~~ a sequence coding for the complete TAR sequence, wild or mutated, or by a fragment of the latter comprising ~~a sequence coding for the~~ SEQ ID No. 1 sequence.

~~According to a particular embodiment of the method of the invention this~~ The nucleic acid coding for the sequences ~~comprising~~ may comprise the sense and antisense sequences of an RNAi of interest separated by the separating sequence described above can be introduced into the cell in the form of an expression vector, particularly in the form of a plasmid or a viral vector.

~~In this embodiment~~ that instance, the RNAi of interest is transcribed from the vector coding for shRNA that ~~will~~ gives rise to the siRNA after cleavage of the single-stranded separating sequence. Thus, the vector comprises at least one nucleotide sequence coding for the sense and antisense sequences of the siRNA separated by a nucleotide sequence comprising at least ~~the sequence~~ referenced under number SEQ ID No. 1 sequence under the control of a transcription promoter.

~~This~~ The nucleic acid described above permits ~~the~~ implementation of the method for the induction of the activity of an RNAi in eukaryote cells.

Moreover, the vector can comprise an antibiotic resistance gene. ~~According to the invention~~ the The antibiotic resistance gene is ~~advantageously~~ may be a neomycin resistance gene, for example.

Kindly replace paragraphs [0028] through [0037] with the following:

~~According to the invention, when~~ When the cells are cells in culture the TAT protein can be introduced into these cells by a simple culturing of the cells in an environment comprising the TAT protein. In fact, ~~an~~ one advantage ~~of the present invention~~ resides in the fact that the TAT protein penetrates substantially spontaneously into the cells when they are cultivated in a culture environment containing the TAT protein. The action of siRNA comprising the TAR separating sequence as previously described can therefore be readily blocked by cultivating the cells containing the siRNA in a culture environment containing the TAT protein and to stimulate the activity of the RNAi by

cultivating these cells in a culture environment without TAT protein, which results in ~~the~~ degradation of the target RNA by the RNAi.

Under these conditions, the culture environment can contain the TAT protein at a concentration comprised between about 0.1 µg/ml and about 1.5 µg/ml and very preferentially about 1 µg/ml.

The TAT protein can also be introduced into the cell in the form of an inducible expression vector comprising a nucleotide sequence coding for the TAT protein. In ~~this~~that instance, the expression of the protein can be induced, which has as a consequence the inhibiting ~~of~~ the activity of the RNAi and therefore ~~the blocking of the~~ degradation of the target RNA by the RNAi. ~~The degradation~~Degradation of the target RNA by the RNAi can then be induced when ~~the~~ synthesis of the TAT protein is itself blocked-itself.

~~According to the invention the~~The cells transfected with the nucleic acid ~~of the invention~~ aremay be mammalian cells. The method applies ~~equally well to the~~ transfection of cells in culture as well as directly in the animal.

The ~~invention~~method permits genes, especially human genes, to be analyzed in a reliable manner from a functional point of view in cells in culture or in animals, especially in mice.

The ~~invention~~method also relates to cells or a cell line in which a nucleic acid ~~in accordance with the invention~~ as previously described was introduced.

The ~~invention~~method also relates to animals comprising cells in which the nucleic acid ~~in accordance with the invention~~ as previously described was introduced.

Finally, ~~the invention has~~this disclosure includes compositions, especially pharmaceutical compositions, as ~~subject matter~~ comprising as an active substance at least one nucleic acid ~~in accord-~~

~~ance with the invention~~ as previously described or cells containing ~~this~~the nucleic acid such as previously described, ~~possibly~~optionally associated in the composition with a compatible excipient.

Other advantages and features of the ~~invention~~method and composition will appear from the following examples in which reference is made to the attached drawing in which ~~figure~~Fig. 1 represents the inhibition of the GFP marker by RNAi.

Kindly replace paragraphs [0040] through [0042] with the following:

Thus, the sequences coding for the sense and antisense siRNA are separated by a minimal TAR loop coded by the separating sequence ~~called~~-SEQ ID No. 1 coding for the minimal sequence recognized by the TAT protein (SEQ ID No. 4).

Coding for the siRNA:

5'	GCAAGCTGACCCTGAAGTTCATCTGAGCTCTGA	3'
	ACTTCAGGGTCAGCTTGC	
	CGTTCGACTGGGACTTCAAGTAGACTCGAGACTTGAAGTCCCAGTCGAACG	

Example 2: Inhibition of the expression of GFP by the siRNAGFP expressed by the vector of ~~example~~Example 1:

Mammalian cells COS-7 are transfected with polyfect (Qiagen) with 4 µg of expression vectors of the siRNA (pTATOF-siRNAGFP, part A, or pSuper siRNAGFP without TAR loop, part B) as well as an expression vector of Green Fluorescent Protein or GFP (500 ng). Sixty hours after ~~the~~ transfection, a Western blot was performed from the total extracts using an antibody directed against the GFP (Santa cruz) or cellular tubulin (Sigma) in order to evaluate the quantity of proteins used for this test.

The cells are cultivated in a DMEM (Gibco) environment containing 10% of fetal bovine serum in the presence (wt) or absence (-) of a vector for the expression (0.5 µg) of TAT protein or of its mutants, co-transfected (V. Bres et al., *Nat. Cell Biol.* Aug. 2003; 5(8): 754-61, the subject matter of which is incorporated by reference).

Kindly replace paragraph [0044] with the following:

Figure Fig. 1 shows the results of this experiment:

Part A:

In the absence of TAT protein and of pTATOF-siRNAGFP, the GFP is present in the cells (column 1).

In the absence of TAT protein and in the presence of the of pTATOF-siRNAGFP vector, the GFP is degraded (column 2).

In the presence of wild TAT protein and of the of pTATOF-siRNAGFP vector the GFP is present in the cells (column 3) in a quantity comparable to that present in the cells that received neither TAT protein nor pTATOF-siRNAGFP vector (column 1) and is therefore not degraded.

In the presence of mutated TAT protein and of the of pTATOF-siRNAGFP vector the GFP is degraded in the cells (columns 4, 5).

Part B:

In the absence of TAT protein and of pSupersiRNAGFP vector (without TAR sequence) the GFP is present in the cells (column 1).

In the absence of TAT protein and in the presence of the pSupersiRNAGFP vector the GFP is degraded (column 2).

In the presence of wild or mutated TAT protein and of the pSupersiRNAGFP the GFP is degraded in the cells (columns 1, 4 and 5).